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GRANT NUMBER DAMD17-94-J-4056

TITLE: Vaccines to Breast Cancer Based on p53 Mutants

PRINCIPAL INVESTIGATOR: Hildegund C. J. Ertl, M.D.

CONTRACTING ORGANIZATION: The Wistar Institute
Philadelphia, PA 19104

REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (1 Sep 95 - 31 Aug 96)

4. TITLE AND SUBTITLE Vaccines to Breast Cancer Based on p53 Mutants

5. FUNDING NUMBERS
DAMD17-94-J-4056

6. AUTHOR(S)
Hildegund C. J. Ertl, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Wistar Institute
Philadelphia, PA 19104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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13. ABSTRACT *(Maximum 200*

P53 a tumor suppresser protein is commonly overexpressed and/or mutated in human breast cancer cells. We generated a number of different vaccine prototypes based on recombinant viruses, i.e., vaccinia or adenovirus or plasmid vectors expressing wild-type or mutant mouse p53 to test if they could induce immunity to tumors expressing wild-type or mutant p53. Experiments thus far have shown that vaccines expressing wild-type p53 induce partial protection against the growth of a tumor also expressing wild-type p53. The efficacy of the vaccine could be augmented by additional treatment with Interleukin 12 given after challenge with tumor cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer`	14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	limited

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INTRODUCTION

The goal of our research is to test vaccines to p53 for the induction of protective immunity to tumor cells overexpressing p53 or expressing mutated p53. p53 is a tumor suppresser protein that controls cell growth. Mutations of p53 which cluster in well-defined hot spots of the gene. They generally result in overexpression of the p53 protein due to an extension of its half life are the most commonly found abnormality in human cancer. Up to 90% of human breast cancer carry p53 mutations. Mutated as well as overexpressed proteins can potentially serve as target antigens for immunosurveillance. Tumor cells carrying such proteins commonly fail to directly induce an immune response that is sufficiently efficacious to cause their elimination. Nevertheless immune responses induced by vaccines can recognize tumor cells that carry the appropriate antigen and thus limit their spread. Within the realm of this application we are constructing a number of different vaccine prototypes expressing mutated or wild-type p53 to test their ability to induce immune responses in mice which limit the spread of tumor cells carrying p53 mutation or overexpressing p53.

BODY OF WORK

1. Construction of Vaccines: We have generated a number of recombinant viruses expressing wild-type p53 or p53 mutants. We have used one construct carrying a mutation at 135 and a second construct carrying a double mutation - one at 234 and a second one at 168. These 3 different p53 encoding sequences (one wild-type, one with a single mutation, one with a double mutation) were cloned into transfer vectors for vaccinia virus and adenovirus. Vaccinia virus strain Copenhagen recombinants were generated by homologous recombination in Tk- cells; E1 deleted replication-defective adenovirus (human strain 5) recombinants were generated in the E1 expressing 293 cell line. All of the viral recombinants were purified by a plaque assay 2-3 times to ensure preparation of stock virus free of wild-type virus contamination. The recombinant viruses were initially identified by PCR using primers for p53 or by PCR followed by hybridization using a radiolabeled p53 specific probe. Vaccinia virus p53 recombinants were expanded and titrated on HeLa or Tk- cells, adenovirus p53 recombinants were expanded and titrated on 293 cells. We obtained expression vectors with the SV40 promoter for p53 with the 135 and the 234 mutation and a similar expression vector for wild-type p53. As SV40 is a comparatively weak promoter commonly inefficient for use in DNA vaccines we also generated expression vectors with the CMV promoter for p53 wild-type and the 135 mutation of p53 (vectors based on pcDNA3). The different constructs have thus far been tested as follows:

All of the recombinant viruses were tested by PCR. The expression vectors were tested by restriction enzyme analysis. Expression of p53 by the 3 different vaccinia recombinants was confirmed by Western Blot analysis. Similar experiments are underway for the adenoviral recombinants and the expression vectors.

Tumor Cell Lines: We obtained or generated a number of tumor cell lines. We amplified from the tumor cells part of the transcripts encoding the mutational hot spot region of p53 by RT-PCR. The PCR products were sequenced to identify p53 mutations. The following results were obtained:
GL261 (Glioma line): wild-type p53
EL4 (thymoma line) : wild-type p53
B16F10 (melanoma): wild-type p53
CT26 : wild-type p53
RAG-2 spontaneous tumor: wild-type p53
4102-PRO: UV-induced tumor, obtained from Dr. H. Schreiber): 245 mutation
Meth A Balb/c tumor (obtained from Dr. L. Old and Dr. A. DeLeo, 1 & 2): variable mutations at 234, 168 and 132.

The MethA tumor cells in several experiments gave variable results for the p53 sequence. In order to determine if this was caused by differences of p53 on the 2 alleles or by the presence of a mixture of different cells with distinct mutations in the cell line we cloned the p53 RT-PCR product into the pCR2.1 plasmid and then sequenced a number of individual plasmid clones (~ 12). The results of this experiment are shown in Table 1.

Table 1[1]

Clone #	1	2	3	4	5	6	7	8	9	10	11	12
aa side												
132	wt	wt	wt	mu	wt	wt	mu	wt	wt	mu	wt	wt
168	mu	wt	mu	wt	mu	mu	wt	wt	mu	wt	wt	mu
234	mu	mu	mu	wt	mu	mu	mu	wt	mu	wt	wt	mu

[1] This table contains unpublished data

wt - wild type, mu - mutation

This result clearly showed that the Meth A cell line consisted of a mixture of cells with distinct p53 mutations. The cell line was subcloned and p53 transcripts were isolated from 40 of the subclones. A high percentage (~50%) of the tumor cell subclones lacked p53 message altogether. One of these subclones was maintained to serve as a control for future experiments. The RT-PCR products of the other tumor cell subclones that contained p53 transcripts were sequenced (result see Table 2).

Table 2

No of clones	19*	16	1	1	2	1
aa side						
132	0	wt	wt	mu	?	wt
168	0	mu	wt	wt	wt	mu
234	o	mu	wt	wt	wt	?

wt - wild type, mu - mutation, ? ambiguous sequence, *subclones which lacked p53 transcripts

One of the tumor cell subclones containing mutations at 168 and 234 was expanded and is currently being titrated in mice. Preliminary data indicate that the subcloned line growth less rapidely in mice compared to the parenteral line. The 4102-PRO cell line is also being titrated in mice. To test if tumor cells with normal p53 could be treated with a vaccine to p53 we also titrated the GL261 cell line in C57Bl/6 mice. An initial tumor burden of 2 - 5 x 10^5 cells given subcutaneously was shown to result in clearly visible tumors in >90% of mice within 14 days.

We also isolated spontaneously arising tumors from p53 knock-out mice (3) (for transfection with an expression vector encoding mutated p53), we induced tumors with MethA in C57Bl/6 mice and p53 knock-out mice. Thus far 4 out of 10 C57Bl/6 mice and one p53-KO mouse developed tumors which we are trying to establish as cell lines.

Protection Experiments: We have thus far only conducted protection experiments with the GL261 cell line which expresses wild-type p53 (4). In the initial experiments we used p53

knock-out mice as well as C57Bl/6 mice assuming that the knock-out mouse strain that also has the C57Bl/6 background would develop immunity to p53 more readily compared to wild-type mice. This was not consistently the case.

Vaccinia virus recombinant: Both mouse strains were initially immunized with the vaccinia recombinant virus expressing wild-type p53 (termed Vap53-wt) or as a control with a vaccinia virus recombinant expressing the glycoprotein of rabies virus (VRG). In one experiment naive mice were used as a control. Data of these experiments are shown in Table 3.

Table 3[2]. Protection against tumor challenge after vaccination with Vacp53-wt.

Mice	Tumor cells	Vaccine/pfu	# Mice (tumor bearing/total)					% Protection
Exp. #		days postinoculation:	7	14	21	28	42	
I								
C57Bl/6	2 x 10^5 GL261	none	4/5	5/5	5/5	5/5	5/5	0
C57Bl/6	2 x 10^5 GL261	2 x 10^7 Vacp53-wt	0/5	1/5	2/5	4/5	4/5	20
p53-KO*	2 x 10^5 GL261	2 x 10^7 Vacp53-wt	0/5	0/5	2/5	5/5	5/5	0
II.								
C57Bl/6	1 x 10^6GL261	2 x 10^7 VRG	3/4	4/4	4/4	4/4	4/4	0
C57Bl/6	1 x 10^6GL261	2 x 10^7 Vacp53-wt	0/5	3/8	4/8	5/8	6/8	25
p53-KO	1 x 10^6 GL261	2 x 10^7Vacp53-wt	1/9	4/9	5/9	5/9	5/9	45
III.								
C57Bl/6	2.5 x 10^5 GL261	3 x 10^7 VRG	4/10	5/10	8/10			
C57Bl/6	2.5 x 10^5 GL261	3x10^7Vacp53-wt	0/15	0/15	3/15			
p53-KO	2.5 x 10^5 GL261	3 x 10^7VRG	3/10	5/10	5/10			
p53-KO	2.5 x 10^5 GL261	3 x 10^7 Vacp53-wt	0/10	2/10	3/10			

Groups of mice were vaccinated with different doses of Vacp53-wt (2 x 10^7 or 3 x 10^7 pfu). Control mice were left untreated (none) or were vaccinated with the VRG recombinant, a vaccinia virus recombinant expressing the rabies virus

[2] This table contains unpublished data

glycoprotein. The results show the number of tumor bearing mice compared to the number of mice of the experiment over an observation time 42 days. Experiment III is currently in progress. * JTrp53TmTY - p53-KO (knock-out) mice.

Using a fairly low dose of the vaccinia recombinant we obtained some protection of C57Bl/6 mice and p53 knock-out mice with regard to the initial appearance of visible tumors. More mice were completely protected against the development of tumors compared to mice immunized with the control construct or nothing. Mice that were protected in the first experiment were challenged 6 weeks later with an increased dose of tumor cells (2 x 10^7) and none of them developed tumors thus demonstrating that the combination of vaccine and subsequent challenge had induced immunological memory in these mice. The results of the experiment is shown in Table 4.

Table 4[3]. Long-lasting protection against rechallenge with a high dose of tumor cells.

Exp. #	Mice	Tumor cells	Morbidity (tumor bearing mice/total mice)
I	C57Bl/6	5 x 10^6GL261	0/1
II	C57Bl/6	5 x 10^6 GL261	0/2
	JTrp53TmTY	5 x 10^6 GL261	0/4

Tumor-free mice from the Experiment in Table 3 received a subsequent challenge with higher dose of tumor cells (5 x 10^6) without additional boost with recombinant vaccinia virus. All control animals, i.e., normal and p53-knockout mice (10/10) developed tumors after 7-10 days. In contrast, all animals vaccinated previously with Vacp53-wt remained tumor-free over the observation time of 55 days.

Adenovirus recombinant: In subsequent experiments C57Bl/6 mice were immunized with the adenoviral recombinant expressing mutant (135) p53. At that time the adenoviral construct expressing wild-type p53 was not yet available to us. As shown in table 5 this recombinant also induced partial protection against challenge with the GL261 tumor.

[3] This table contains unpublished data

Table 5[4]. Protection against tumor challenge after vaccination with Ad5CMVp53$_{135}$.

Mice	Tumor cells	Vaccine/ pfu	# Mice (Tumor bearing/total)					% Protection
		days postinoculation:	7	14	21	28	42	
C57Bl/6	1.5 x 10^5 GL261	3x10^8 Adrab.gp	0/8	1/8	5/8	5/8	6/8	25
C57Bl/6	1.5 x 10^5 GL261	3x10^8 Adp53$_{135}$	0/20	2/20	5/20	6/20	9/20	55
JTrp53TmTY	1.5 x 10^5 GL261	3x10^8 Adrab.gp	0/6	0/6	3/6	4/6	4/6	34
JTrp53TmTY	1.5 x 10^5 GL261	3x10^8 Adp53$_{135}$	0/5	0/5	2/5	2/5	3/5	40

Combination treatment with vaccine and IL-12. In neither of these experiments protection to tumor growth upon vaccination was complete. We thus tested a combination of vaccination with the vaccinia p53 recombinant virus combined with murine IL-12 given either before or after challenge. In a series of experiments mice were vaccinated first with vaccinia recombinants, 2 weeks later they were challenged with tumor and then at different days after tumor inoculation they were injected intraperitoneally with 0.25 μg of IL-12 given daily for 5 days. Three different time schedules for the administration of IL-12 were tested. IL-12 by itself (i.e., in combination with the control vaccine) protected if given early (day 2-5) after tumor challenge. IL-12 given later (i.e., day 5-9) after tumor challenge was more efficacious in mice vaccinated with the p53 expressing vaccine (5). In groups that were treated with IL-12 after 15 days protection was markedly reduced. These data indicate that IL-12 alone has an effect on tumor growth if given early but that better protection is achieved with a combination of IL-12 and a specific vaccine.

[4] This table contains unpublished data

Table 6[5]. Anti-tumor responses in animals treated with vaccine prophylactically and IL-12 after challenge

Tumor cells	Vaccine	IL-12 treatment 0.25 µg daily/ days postinoculation	# Mice (tumor bearing/total) (% Protection)	
1.5 x 10^5 GL261	2 x 10^7 VRG	none	4/6	(33%)
"	"	days 2-5	0/8	(100%)
"	"	days 5-9	3/8	(38%)
"	"	days 15-19	3/8	(38%)
1.5 x 10^5 GL261	2 x 10^7 Vacp53-wt	none	3/8	(38%)
"	"	days 2-5	0/8	(100%)
"	"	days 5-9	0/8	(100%)
"	"	days 15-19	2/7	(29%)

Post-challenge treatment with vaccine and IL-12: We next tested if a combination treatment of vaccine and IL-12 could eliminate already established tumors. Mice were injected subcutaneously with 1.5 x 10^5 tumor cells. Groups of 10 mice selected for the experiment bearing large established tumors (diameter 0.3-0.8 mm) 30 days later were immunized with 3 x 10^7 pfu Vacp53-wt or with the control construct, i.e., VRG. The systemic administration of rIL-12 was initiated 3 days after vaccination by giving 5 intraperitoneal injections of 25 µg of rIL-12 to tumor bearing and vaccinated mice (6).
In the control group the progressive growth of tumors was inhibited for some time by the IL-12 treatment, no cures were observed and tumors eventually started growing again, resulting in death of the animals. (Mice with an overly large tumor burden were euthanized for humanitarian reasons). In contrast, in some of the mice treated with IL-12 and immunized with Vacp53-wt complete tumor regression was observed. These results suggest that IL-12 in combination with recombinant vaccinia expressing p53 oncoprotein has a therapeutic effect on already established tumors expressing wild-type p53.

[5] This table contains unpublished data

Table 7[6]. Combined therapeutic effect of Vacp53-wt and IL-12 on day 2 post-vaccination on established tumors.

Tumor cells	Vaccine / pfu	tumor incidence			% Complete remission
	days after IL-12 treatment:	1	30	40	
1.5 x 10^5 GL 261	3 x 10^7 VRG	5/5	5/5	5/5	0
1.5 x 10^5 GL 261	3 x 10^7 Vacp53-wt	10/10	5/10	5/10	50

CONCLUSION: Data obtained so far clearly show that wild-type p53 present in tumor cells can serve as a target for immunosurveillance. Partial protection to tumor cells expressing p53 could be achieved by pre-vaccination with a vaccinia recombinant and an adenoviral recombinant expressing this protein. Protection was improved by additional treatment with IL-12 given early after tumor challenge. The combination of vaccine and IL-12 was shown to also result in complete regression of already established tumors.

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